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(54) Title: MODULATION OF GENE EXPRESSION BY MODULATING HISTONE ACETYLATION

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(57) Abstract: The present invention provides a composition, which contains a first agent that affects histone acetylation and a second agent that induces gene expression through a phosphorylation dependent response element binding factor. The invention also provides a method of modulating the phosphorylation state of a response element binding factor by contacting a cell containing the binding factor with an agent that affects histone acetylation, thereby modulating the phosphorylation state of the response element binding factor. The present invention further provides a method of ameliorating a pathologic condition by potentiating expression of a gene regulated by a phosphorylation dependent response element binding factor in a subject, by administering to the subject a pharmaceutical composition containing a first agent that affects histone acetylation and a second agent that induces expression of the gene through the phosphorylation dependent response element binding factor. The invention also provides a method of altering expression of a nucleosomal organized nucleic acid molecule by modulating attenuation of expression of the nucleic acid molecule, which contains an expressible nucleotide sequence operably linked to an inducible response element, which is bound in a phosphorylation dependent manner by a response element binding factor. Also provided is a method of identifying an agent that modulates attenuation of expression of a nucleic acid molecule.

MODULATION OF GENE EXPRESSION BY MODULATING HISTONE ACETYLATION

FIELD OF THE INVENTION

The present invention relates generally to the field of molecular biology and gene regulation, and more specifically to compositions and methods for modulating
5 the regulation of expression of a nucleosomal organized nucleic acid molecule from an inducible response element.

BACKGROUND OF THE INVENTION

The regulation of gene expression is a tightly controlled process. For
10 example, certain genes are expressed only for a few days during development, then are turned off and never again expressed in a normal, healthy individual. Other genes are active during certain stages of the cell cycle, for example, immediately preceding mitosis, but otherwise are not expressed. Still other genes are expressed only in response to a physical, chemical or environmental stimulus to a cell containing the
15 gene. Such inducible genes generally are active for a short period of time, thus allowing the cell to produce a protein that, for example, protects it from the stimulus. When the stimulus is removed, expression of the gene is turned off or reduced to a basal level, until a stimulus again contacts the cell.

20 In general, upon contact of a cell with a stimulus, transcription of an inducible gene that is responsive to the stimulus begins, increases to a maximal level, then decreases back to the pre-induced level. In some cases, for example, where the stimulus is persistent, the time may be extended before the level of transcription decreases. Ultimately, however, transcription decreases from a maximal level to the
25 pre-induced level, which may be a low level of expression or no expression at all.

- In many disease states, including congenital diseases and infectious diseases, and even due to the process of aging, regulation of gene expression can be removed from the otherwise tight control mechanism. As a result, the genes may be over-expressed, for example, as occurs in various cancers, or the genes may lose or
- 5 have a decreased ability to respond to a stimulus. Some of the biochemical effects observed in patients having dementias such as Alzheimer's disease, for example, have characteristics suggestive of errors in the regulation of molecules associated with learning and memory. In particular, evidence suggests that some genes are not responsive to stimuli in the same way that they would be in a healthy individual. For
- 10 example, the genes may not be induced to as high a level as normally would occur, or the genes may be turned off earlier than normal. As a result, a sufficient amount of a gene product necessary to normal growth and survival of the cell may not be produced.
- 15 Various methods, including gene therapy, are being developed for treating pathologic conditions that are associated with inappropriately low levels of gene expression. However, such methods have significant drawbacks. For example, while gene therapy can be effective in some cases, often the genes that are introduced into a cell become integrated in the chromosomal DNA in such a way that they cannot be
- 20 expressed. In other cases, the genes are expressed for a short period of time, but then become refractory to stimulation. Unfortunately, few if any effective methods have been developed for prolonging gene expression in a cell. Furthermore, no effective method has been described for increasing the expression of a gene that is expressed at a decreased level due to a pathologic condition such as Alzheimer's disease, or as
- 25 occurs during aging. Thus, a need exists to identify agents that can modulate the regulation of gene expression. The present invention satisfies this need and provides additional advantages.

SUMMARY OF THE INVENTION

- 30 The present invention relates to compositions, which contain at least one agent that affects histone acetylation and at least one agent that induces expression of a gene through a phosphorylation dependent response element binding factor. For example, a composition can contain at least one histone deacetylase (HDAC) inhibitor and at

least one agent that induces gene expression through a phosphorylation dependent response element binding factor. In one embodiment, the HDAC inhibitor can be trichostatin A, a butyrate compound, trapoxin, an anti-HDAC antibody, or an HDAC binding fragment of an anti-HDAC antibody. In another embodiment, the response 5 element binding factor can be a cyclic adenosine 3', 5'-monophosphate (cAMP) response element binding factor (CREB). In still another embodiment, the composition is a pharmaceutical composition.

The agent that affects histone acetylation can be an agent that inhibits the 10 expression or suppresses the activity of an HDAC or an HAT, or can be an agent that induces the expression of or stimulates the activity of an HDAC or an HAT. The agent that induces gene expression through a phosphorylation dependent response element binding factor can be a second messenger, for example, cAMP or a derivative thereof, or a molecule that induces expression of a second messenger, for example, 15 forskolin, which induces cAMP expression, or can be a calcium ion, a neurotrophic factor, a growth factor, a hormone, or other physical, chemical or environmental agent; and can be, for example, an agent that induces activity of a protein kinase, which phosphorylates the response element binding factor, or a protein phosphatase, which dephosphorylates the response element binding factor.

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The invention also relates to methods of modulating the phosphorylation state 25 of a phosphorylation dependent response element binding factor by contacting a cell containing the binding factor with an agent that affects histone acetylation. For example, the cell can be contacted with an HDAC inhibitor or a HAT inducer, thereby modulating the phosphorylation state of the response element binding factor. The HDAC inhibitor, for example, can be any HDAC inhibitor such as trichostatin A, a butyrate compound, or trapoxin, and the response element binding factor can be any phosphorylation dependent transcription factor, for example, CREB, a serum response element binding factor, or NF- κ B.

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In one embodiment, a method of modulating the phosphorylation state of the phosphorylation dependent binding factor includes further contacting the cell containing the binding factor with an agent that induces gene expression through the

phosphorylation dependent response element binding factor. The agent can directly or indirectly affect the activity or expression of a protein kinase that phosphorylates the response element binding factor, or of a protein phosphatase that dephosphorylates the response element binding factor, thereby increasing or decreasing the

5 phosphorylation state of the binding factor. For example, the agent can be a second messenger such as cAMP, a cell permeable derivative of cAMP or calcium ions; or an agent that affects the level of a second messenger, such as forskolin, which increases the level of cAMP in the cell, thereby stimulating protein kinase A activity in the cell, which, in turn, can phosphorylate CREB and induce expression of one or more genes

10 containing a cAMP response element. The response element binding factor can be a single polypeptide or can contain two or more polypeptide subunits, and can be, for example, CREB, serum response element binding factor, c-jun, v-jun, c-fos, v-fos, Elk, STAT1 (signal transducer and activator of transcription-1), NFAT (nuclear factor of activated T lymphocytes), a TCF (ternary complex factor), E2F, DP1; C/EBP

15 (CCAAT enhancing binding protein), an ATF (activating transcription factor) such as ATF-1, NF- κ B or another Rel family member such as a RelA containing binding factor, or the like.

The present invention further relates to methods of ameliorating a pathologic condition by altering the expression of a gene regulated by a phosphorylation dependent response element binding factor in a subject, by administering to the subject a pharmaceutical composition of the invention. Thus, a method of the invention, for example, can be a method of potentiating expression of a gene regulated by a phosphorylation dependent response element binding factor in a

20 subject, by administering to the subject a pharmaceutical composition containing an HDAC inhibitor or an HAT inducer, and an agent that induces expression of the gene through the phosphorylation dependent response element binding factor. The pathologic condition, which can be ameliorated due to increased or decreased expression of the particular gene, can be a neuropathology that results in a motor

25 disorder, for example, Parkinson's disease; a neuropathology that results in a cognitive disorder, for example, Alzheimer's disease; a neuromuscular pathology, for example, amyotrophic lateral sclerosis; a cardiac dysfunction; renal dysfunction; endocrine disorder; dysfunction of gluconeogenesis; immunosuppressive condition;

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osteopathologic condition; dermal condition; or other pathologic condition amenable to treatment by potentiating expression of gene that is regulated by a phosphorylation dependent response element binding factor.

5 The invention also relates to methods of altering expression of a nucleosomal organized nucleic acid molecule by modulating attenuation of expression of the nucleic acid molecule, which contains an expressible nucleotide sequence operably linked to an inducible response element that is regulated due to binding of a response element binding factor in a phosphorylation dependent manner. A method of the
10 invention can be performed, for example, by using an HDAC inhibitor to inhibit deacetylation of the histones involved in forming the structure of the nucleosomal organized nucleic acid molecule, thereby inhibiting attenuation of expression of the nucleic acid molecule and increasing its expression. Histone deacetylation can be inhibited by inhibiting the activity of an HDAC, using an HDAC inhibitor such as
15 trichostatin A, a butyrate compound, or trapoxin. A method of the invention also can be performed, for example, by using a HAT inhibitor to inhibit histone acetylation, thereby enhancing attenuation of expression of the gene and decreasing expression of the nucleic acid molecule.

20 The invention further relates to a method of identifying an agent that modulates attenuation of expression of a nucleic acid molecule, by contacting a test agent suspected of having such modulating activity, a nucleosomal organized nucleic acid molecule containing an expressible nucleotide sequence operably linked to an inducible response element that is regulated by a phosphorylation dependent binding
25 factor, and an inducing agent; and detecting a difference in attenuation of expression of the nucleic acid molecule in the presence of the test agent as compared to attenuation in the absence of the test agent, wherein detecting a difference identifies the test agent as an agent that modulates attenuation of expression of the nucleosomal organized nucleic acid molecule. In general, an agent identified by a method of the
30 invention does not substantially modulate attenuation of expression of the nucleic acid molecule when it is not in a nucleosomal organized form, and the agent does not substantially affect early burst phase transcription of the nucleic acid molecule. In one embodiment, the agent that modulates attenuation of expression can be an HDAC

inhibitor or an HAT inducer, each of which inhibits the attenuation of expression of the nucleic acid molecule, thereby potentiating expression of the nucleic acid molecule. In another embodiment, the agent can be an HDAC inducer or a HAT inhibitor, which can enhance attenuation of expression of the nucleic acid molecule,
5 thereby suppressing expression of the nucleic acid molecule.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions, which contain a first agent that affects histone acetylation, and a second agent that induces gene expression through a
10 phosphorylation dependent response element binding factor. Compositions of the invention are useful for increasing or decreasing the level of expression of inducible gene(s) by modulating the attenuation phase of transcription of the gene(s). In particular, the compositions and methods of the invention are useful for affecting expression of gene(s) or other nucleosomal organized nucleic acid molecule
15 containing inducible response element(s). As such, the invention also provides methods of ameliorating a pathologic condition susceptible to treatment by modulating the expression of a gene that is regulated by a phosphorylation dependent response element binding factor, and methods of identifying agents that can modulate the attenuation of gene expression.

20

A composition of the invention is exemplified by a first agent that is a histone deacetylase (HDAC) inhibitor and a second agent that induces gene expression through a phosphorylation dependent response element binding factor. Such a composition of the invention is useful, for example, for potentiating gene expression
25 by inhibiting the attenuation phase of gene expression. Although reference is made herein to a "first agent" and a "second agent," such designations are for convenience of discussion only and do not indicate, for example, a ranking or other significance.

Many signal transduction pathways promote cellular gene expression with
30 burst-attenuation kinetics such that maximal rates of transcription are achieved within 30 minutes of stimulation, and return to baseline levels after about 2 to 4 hours (Sasaki et al., *J. Biol. Chem.* 259:15242-15251 (1984); each of the references cited in the disclosure is incorporated herein by reference). The induction of gene expression

by cyclic adenosine 3', 5'-monophosphate (cAMP), which stimulates gene expression via protein kinase A (PKA) mediated phosphorylation of cAMP response element binding protein (CREB) at Ser133, is an example of such a signal transduction system. In the cAMP signal transduction system, CREB Ser133 phosphorylation 5 promotes recruitment of the co-activator CREB binding protein (CBP) and its paralog P300. CBP and P300 are histone acetyl transferases (HATs) that have been proposed to mediate target gene activation, at least in part, by destabilizing promoter bound nucleosomes, thereby allowing assembly of the transcriptional apparatus.

10 Transcriptional activation via the second messenger cAMP is rate limited by nuclear entry of PKA catalytic subunit, a passive process that plateaus after 15 to 30 minutes and coincides with peak levels of CREB Ser133 phosphorylation and target gene activation (Hagiwara et al., Cell 70:105-113 (1992); Hagiwara et al., Mol. Cell Biol. 13:4852-4859 (1993)). Over the subsequent 2 to 4 hour attenuation phase, 15 transcription rates return to pre-stimulus baseline levels due, in part, to protein phosphatase PP-1 mediated dephosphorylation of CREB at Ser133 (Hagiwara et al., *supra*, 1992).

20 It has been suggested that the paralogous co-activators CBP and P300 mediate target gene activation during the burst phase by acetylating promoter bound nucleosomes, allowing productive assembly of the transcriptional apparatus (Bannister and Kouzarides, Nature 384:641-642 (1996); Ogryzko, Cell 953-959 (1996)). In cellular microinjection experiments, in which endogenous CBP activity was sequestered using an anti-CBP antiserum, HAT defective forms of CBP were 25 unable to rescue CREB dependent target gene activation (Korzuś et al., Science 279:703-7093 (1998)). Recent studies in other signaling systems have reinforced the notion that chromatin remodeling may be a prerequisite for induction of signal dependent genes.

30 P300 has been reported to promote target gene activation via the estrogen receptor *in vitro* on chromatin assembled nucleic acid templates, but not on non-chromatinized templates (Kraus and Kadonaga, Genes Devel. 12:331-342 (1998)). Moreover, stimulation of the interferon β promoter *in vivo* was accompanied

by nucleosome acetylation over the promoter; and mutations in promoter bound factors that abrogated recruitment of CBP correspondingly inhibited both nucleosome acetylation and target gene activation (Parekh and Maniatis, Mol. Cell 3:125-129 (1999)). P300 and CBP also interact with other transcription factors, including AP-1, 5 YY-1 and SP-1, and recruitment to a subset of promoters by these factors may confer specificity on their activity (see Jin and Scotto, Mol. Cell. Biol. 18:4377-4384 (1998)).

In addition to its effects on nucleosome remodeling, CBP also can promote 10 target gene expression by associating with RNA polymerase II (RNAPII) complexes (see, for example, Kim and Maniatis, Proc. Natl. Acad. Sci., USA 95:12191-12196 (1998); Nakajima et al., Cell 90:1107-1112 (1997a); Nakajima et al., Genes Devel. 11:738-747 (1997b)). Such CBP:RNAPII complexes appear to be competent for mediating target gene activation via phospho (Ser133) CREB on naked DNA and on 15 nucleosome assembled templates, suggesting that chromatin derepression may not be a prerequisite for target gene activation in response to cAMP (Mayall et al., Genes Devel. 11:887-899 (1997)). In view of these two differing, though not mutually exclusive, models of CBP/P300 function, i.e., a chromatin remodeling model and a RNAPII recruitment model, an investigation of a role of cellular HAT activity for 20 transcriptional induction via CREB was initiated.

As disclosed herein, agents such as HDAC inhibitors, which affect histone acetylation, cooperate with inducing agent generated signals, such as second messenger generated signals, on chromosomal templates, but not on 25 non-chromosomal templates, to increase or decrease inducible expression of a target gene. Remarkably, HDAC inhibitors potentiated gene expression without significantly affecting target gene activation during the early burst phase, for example, when CBP/P300 recruitment to the promoter is maximal (see Example II). Instead, HDAC inhibitors potentiated transcription from cAMP responsive genes during the 30 later attenuation phase by prolonging the time of CREB Ser133 phosphorylation, in a chromatin dependent manner, thereby extending the time that CREB can engage the transcriptional machinery via its association with CBP/P300 (Examples II and IV). These results demonstrate that chromatin bound activators can be differentially

phosphorylated in response to cellular signals depending, in part, on local chromatin structure.

It has been reported that the HDAC inhibitors, TSA and butyrate, can directly
5 affect transcription of the multiple drug resistance (MDR) gene in cells (Jin and
Scotto, *supra*, 1998). However, those studies were performed in the absence of any
inducing agent and, therefore, are distinguishable from the present invention, whereby
HDAC inhibitors modulate the attenuation of induced gene expression. It also has
been reported that TSA can induce a stress response, resulting in increased DNA
10 binding of AP-1 and NF- κ B in Neuro-2a neuroblastoma cells and cultured cerebellar
granule neurons, and increased DNA binding of CREB in the Neuro-2a cells, but not
the cerebellar cells (Salminen et al., *Mol. Brain Res.* 61:203-206 (1998)). Thus, these
results describing that HDACs can induce a stress response, which results in increased
DNA binding of transcription factors, are distinguishable from the present invention,
15 which discloses that HDACs can modulate the attenuation of induced gene
expression.

As used herein, the term "attenuation phase" refers to the stage of gene
expression during which the level of induced gene transcription has begun decreasing.
20 Similarly, the term "attenuation," when used in reference to induced gene expression,
means a decrease in the level of transcription of a gene from the level achieved during
the early burst stage. The time at which the attenuation phase of expression begins
following induction and the length of the attenuation phase can vary for different
genes, depending, for example, on the particular inducing agent, response element, or
25 cognate response element binding factor, including the concentrations of these
components. The attenuation phase of a cAMP responsive gene, for example, begins
about 15 to 30 minutes after induction of transcription and proceeds for about 2 to 4
hours, during which the level of transcription returns to a basal or near basal level.
Methods for determining the time of onset and the length of an attenuation phase for a
30 particular system are disclosed herein, including, for example, run-on transcription
assays and northern blot analysis, and other methods known in the art (see, for
example, U.S. Patent No. 5,191,646).

As used herein, the term "inhibiting attenuation" means reducing or inhibiting the decrease in gene expression that normally occurs for a particular transcription system. Thus, an agent that inhibits attenuation of gene expression potentiates gene expression such that expression remains at a higher level or for a longer time than it 5 otherwise would in the absence of the agent. Conversely, the term "enhancing attenuation" means increasing the decrease in gene expression that normally would occur in a particular system. As such, an agent that enhances attenuation of gene expression suppresses gene expression such that less of a gene product, for example, would be produced than would otherwise be produced in the absence of the agent.

10

As used herein, the term "response element" means a nucleotide sequence that, when operably linked to an expressible nucleotide sequence, confers an ability to regulate expression of the linked nucleotide sequence. Response elements are well known in the art and include, for example, cAMP response elements (CRE; 15 Andrisani, Crit. Rev. Eukaryotic Gene Expr. 9:19-32 (1999)), phorbol ester response elements (TRE; Angel et al., Cell 49:729-739 (1987)), serum response elements (SRE), a κ response element such as a κB element (Sen and Baltimore, Cell 46:705-709 (1986)), and the like. Response elements often present a symmetry, which can facilitate binding by a dimeric binding factor. For example, the κB response element 20 exhibits dyad symmetry, which may facilitate cooperative binding by a dimeric NF-κB binding factor. In addition, response elements can have increased activity when present in two or more copies operably linked to an expressible nucleotide sequence.

25 The term "operably linked" is used herein to mean that one or more response elements are appropriately positioned with respect to an expressible nucleotide sequence, as well as to any other regulatory element such as a promoter or the like, that is contained in the nucleic acid molecule comprising the response element, such that the response element confers its regulatory function upon the expressible 30 nucleotide sequence. The term "expressible nucleotide sequence" is used herein to mean any nucleotide sequence that can be operably linked to a response element and transcribed into an RNA. In general, an expressible nucleotide sequence encodes at least one gene product that can provide a desirable effect to a cell, which can be in

tissue culture or in a subject, or that can be useful as a reporter molecule for detecting modulation of the attenuation phase of expression from the response element.

- Expression of the expressible nucleotide sequence can be detected, for
- 5 example, by a hybridization method, which utilizes a specifically hybridizing nucleic acid sequence. Such a hybridization method can, but need not, be followed by a polymerase chain reaction or other amplification method. Expression of the expressible nucleotide sequence also can be detected by detecting a polypeptide encoded by the nucleotide sequence. Such a reporter polypeptide can be any
- 10 detectable polypeptide such as an enzyme, for example, β -galactosidase or luciferase, the expression of which can be visualized upon contact with its appropriate substrate; an antigen that is recognized by a specific antibody, for example, a FLAG epitope; or any other ligand (or receptor) that is recognized by a specific cognate receptor (or ligand), for example, glutathione S-transferase, which is recognized by glutathione, or
- 15 a polyhistidine peptide, which is recognized by a divalent metal ion such as nickel ion.

- Response elements are characterized, in part, by the ability to bind one or a few specific response element binding factors. For purposes of the present invention,
- 20 the binding of a response element binding factor to its cognate response element depends, in part, on the phosphorylation state of the binding factor. As such, reference is made herein to a "phosphorylation dependent response element binding factors," which is a binding factor that binds to its cognate response element depending on whether one or more specific amino acids in the binding factor is
- 25 phosphorylated. Such phosphorylation dependent response element binding factors include various transcription factors, including polypeptide subunits thereof, for example, CRE binding factor (CREB; U.S. Patent No. 5,919,649), SRF (SRE binding factor), AP-1 (activator protein-1), c-jun, v-jun, c-fos, v-fos, Elk, STAT1 (signal transducer and activator of transcription-1), NFAT (nuclear factor of activated
- 30 T lymphocytes), a TCF (ternary complex factor), E2F, DP1; C/EBP (CCAAT enhancing binding protein), an ATF (activating transcription factor) such as ATF-1, and NF- κ B (nuclear factor- κ B) or another Rel family member such as a RelA containing binding factor.

Phosphorylation dependent response element binding factors often exist as multisubunit proteins. For example, AP1 exists as homodimers or heterodimers of c-jun or c-fos, or their oncogene versions, v-jun or -v-fos, respectively. NF- κ B, 5 which binds the κ B response element, is an example of a phosphorylation dependent response element binding factor. NF- κ B is a member of the Rel family of proteins, which comprise dimeric transcription factors that can contain any of several different polypeptide subunits, and, depending on the polypeptide subunits, variously bind similar response elements. The Rel family of transcription factors, which are present 10 in most if not all animal cells (Thanos and Maniatis, *Cell* 80:629-532 (1995)), include, for example, RelA (p65), c-Rel, p50, p52 and the *Drosophila* dorsal and Dif gene products, and are characterized by a region of about 300 amino acids sharing approximately 35% to 61% homology (Siebenlist et al., *Ann. Rev. Cell Biol.* 10:405-455 (1994)).

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Different Rel protein dimers vary in their binding to different κ response elements, their kinetics of nuclear translocation, and their levels of expression in a tissue (Siebenlist et al., *supra*, 1994). The κ B response element, for example, has been identified in numerous cellular and viral promotors, including promotors present 20 in human immunodeficiency virus-1 (HIV-1); immunoglobulin superfamily genes such as MHC class 1 genes; cytokine genes such as the tumor necrosis factor α , interleukin-1 α (IL-1 α), IL-2, IL-6 and the granulocyte-macrophage colony stimulating factor gene; chemokine genes such as the RANTES and IL-8 genes; and cell adhesion protein genes such as the E-selectin gene. As such, methods of the invention, as 25 disclosed herein, can be used to modulate attenuation of expression of various genes, and can be useful for ameliorating a variety of pathologic conditions that are amenable to treatment by modulating such attenuation.

A composition of the invention contains a first agent that affects histone 30 acetylation and a second agent that induces gene expression through a phosphorylation dependent response element binding factor. As used herein, the term "affects histone acetylation" means increases or decreases the amount of histone acetylation or the time that a histone remains acetylated. It should be recognized that

an agent that affects histone acetylation is identified by comparing the amount of histone acetylation of a particular nucleosomal organized nucleic acid molecule in the presence of the agent with the amount of histone acetylation in the absence of the agent. Agents that affect histone acetylation include HDAC inhibitors and HAT inducers, both of which can increase the amount of histone acetylation or the time histones remain acetylated, and, conversely, HDAC inducers and HAT inhibitors, both of which can decrease the amount or time of histone acetylation. As disclosed herein, agents that increase histone acetylation potentiate gene expression by inhibiting attenuation of expression of the gene. Similarly, agents that decrease histone acetylation suppress gene expression by enhancing attenuation of expression of the gene.

A composition of the invention is exemplified by an HDAC inhibitor and an agent that induces expression of a gene that is regulated by a phosphorylation dependent response element binding factor. More specifically, a composition of the invention contains at least one HDAC inhibitor, and, if desired, can contain two or more HDAC inhibitors, and, similarly, contains at least one inducing agent, but can contain more than one inducing agent. HDAC inhibitors are well known in the art and include, for example, trichostatin A (Yoshida et al., *J. Biol. Chem.* 265:17174-17179 (1990)), butyrate compounds such as sodium butyrate, trapoxin (Yoshida et al., *BioEssays*, 17:423-430 (1995) Itazaki et al., *J. Antibiot.* 43:1524-1532 (1990)), HC-toxin (Liesch et al., *Tetrahedron* 38:45-48 (1982)); chlamydocin (Closse et al., *Helv. Chim. Acta* 57:533-545 (1974)); Cly-2 (Hirota et al., *Agricul. Biol. Chem.* 37:955-56 (1973)); WF-3161 (Umehana et al., *J. Antibiot.* 36:478-483 (1983)), Tan-1746 (Japanese Patent No. 7196686), apicidin and analogs thereof (see, also, U.S. Patent No. 5,922,837, WO/99/36532, WO/98/40080, WO/98/40065, WO/98/39966, WO/9929114, WO/98/00127, WO/97/11366), and the like. Additional agents having HDAC inhibitor activity can be identified using methods as disclosed herein or otherwise known in the art.

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An agent that affects gene expression through a phosphorylation dependent response element binding factor is selected based on the particular system and, depending on the particular system, would be well known to one in the art. For

example, where expression of a gene depends on binding of phosphorylated CREB to a CRE, the inducing agent can be cAMP or a cell permeable cAMP derivative; or forskolin, which induces cAMP expression; or calcium ions, or a neurotrophic factor, growth factor, hormone, or other physical, chemical or environmental stimulus that

5 leads to CREB phosphorylation on Ser133. Similarly, where expression of a gene depends on binding of AP-1 to a TRE, the agent can be a phorbol ester or other agent, which can result in selective phosphorylation of AP-1 via a particular protein kinase such as a protein kinase C, depending on whether the AP-1 to be phosphorylated is, for example, a homodimer of jun polypeptides or fos polypeptides, or a heterodimer

10 of jun and fos polypeptides. It should be recognized that an agent useful in a composition of the invention, or for practicing a method of the invention, can act directly or indirectly to stimulate the activity or induce the expression of a protein kinase that phosphorylates the response element binding factor, or of a protein phosphatase that dephosphorylates the response element binding factor, or to inhibit

15 the activity of a protein kinase or a protein phosphatase involved in affecting the phosphorylation state of the particular response element binding factor.

A composition of the invention can be in a dry form or in solution. Where the composition is in a dry form, it can be contained in a vial or other suitable container

20 and can be maintained in a vacuum or in an air or other environment, for example, stored in a nitrogen environment to reduce the likelihood of an adverse oxidation reaction occurring with the HDAC inhibitor or the inducing agent. Where the composition is in solution, a suitable solvent is selected based on the physicochemical properties of the HDAC inhibitor and the inducing agent. For example, where the

25 HDAC inhibitor and the inducing agent are water soluble, the composition can be an aqueous composition, which can be buffered, can have a selected ionic strength, and can be in any concentration, as desired. The composition also can contain additional components, including, for example, an antioxidant, a stabilizing material, a miscibility agent, or the like.

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A composition of the invention can be useful, for example, as a reagent for affecting gene expression in an experimental system, including an *in vitro* system, a cell based system or an animal model, or can be useful for treating a subject, which

can be an animal such as a mammal, particularly a primate such as a human, having a pathologic condition. When administered to a subject, a composition of the invention is administered as a pharmaceutical composition containing an HDAC inhibitor, an agent that induces gene expression through a phosphorylation dependent response element binding factor, and a pharmaceutically acceptable carrier. A pharmaceutical composition of the invention can be provided in a sterile form, or can be sterilized prior to administration to a subject.

Pharmaceutically acceptable carriers are well known in the art and include, for example, aqueous solutions such as water or physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters. A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize or to increase the absorption of the HDAC or the inducing agent or both. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, in part, on the physicochemical properties of the HDAC and the inducing agent, and on the route of administration of the composition. The pharmaceutical composition also can contain additional substances, as desired, including, for example, nutrients, diagnostic agents, or therapeutic agents.

The invention also provides a method of modulating the phosphorylation state of a response element binding factor by contacting a cell containing a phosphorylation dependent response element binding factor with an HDAC inhibitor. Such a method also can include contacting the cell containing the binding factor with an agent that induces gene expression through the phosphorylation dependent response element binding factor. Depending on the particular phosphorylation dependent response element binding factor, an agent that affects histone acetylation, for example, an HDAC inhibitor or an HAT inducer, can increase or decrease the phosphorylation state of the binding factor.

Nucleosome acetylation is believed to constitute an integral component in the process of target gene activation by extracellular stimuli. As disclosed herein, promoter bound nucleosomes also are involved in modulating the phosphorylation status of nuclear activators (see Examples V and VI). Although no mechanism is proposed herein for this action of nucleosomes, the nucleosomal structure may limit access of signal dependent kinases such that they are unable to maintain phosphorylation of a response element binding factor. By inhibiting deacetylation of the histones that form the structure of a nucleosomal organized nucleic acid molecule, the signal dependent kinase may be able to effect phosphorylation of the binding factor, thereby inhibiting attenuation of expression of the nucleic acid molecule and potentiating its expression.

Three different HDAC inhibitors, butyrate, trichostatin A, and trapoxin, were examined, and each synergized with cAMP agonists to promote somatostatin gene expression on chromosomal, but not extra-chromosomal templates, in a fibroblast line containing chromosomal copies of the somatostatin gene (see Example V). Run-on transcription assays demonstrated that the HDAC inhibitors potentiated somatostatin transcription, primarily during the late attenuation phase, by extending the time that CREB was phosphorylated in response to cAMP (Examples I and II). Remarkably, the initial rate and maximal amplitude of CREB phosphorylation were unaffected by HDAC inhibitors, and the prolongation of CREB phosphorylation was not due directly to enhanced PKA activity or decreased PP-1 activity.

Potentiation of gene expression was dependent on presentation of the target nucleic acid molecule in a chromatin-like form. For example, in transient transfection assays, a GAL4-CREB polypeptide, which lacked the CREB DNA binding domain and, therefore, was unable to bind to the cellular CRE sites, remained heavily phosphorylated throughout the attenuation phase and was unresponsive to HDAC inhibitor (Example V). Similarly, over-expression of a dominant negative A-CREB polypeptide, which displaces CREB from its resident chromosomal sites, strongly enhanced Ser133 phosphorylation of CREB during the late attenuation phase (Example V).

Imaging studies using fluorescent tagged PKA suggested that the catalytic subunit enters the nucleus via passive diffusion in response to cAMP stimulation, and equilibrates throughout the nuclear compartment where it subsequently phosphorylates CREB (Hagiwara et al., *supra*, 1993; Harootunian et al., *Mol. Biol. Cell* 4:993-1002 (1993)). Ser133 phosphorylation does not alter CREB DNA binding activity *per se* (Hagiwara et al., *supra*, 1993; Kwok et al., *Nature* 380:642-646 (1996)); however, reports differ as to whether cAMP induces occupancy of the CRE site by CREB on chromatin (see, for example, Nichols et al., *EMBO J.* 11:3337-3346 (1992); Wolfl et al., *Mol. Endocrinol.* 13:659-669 (1999)). Although there is no hard evidence for an extra-chromosomal pool of CREB that mobilizes to DNA upon PKA phosphorylation, previous estimates of cellular CREB expression levels (5×10^4 copies per cell) do not exclude that possibility (Hagiwara et al., *supra*, 1993).

Based on cellular pulse-chase experiments, which revealed that the half-life of phospho (Ser133) is about 30 minutes, chromatin bound CREB likely undergoes several rounds of rephosphorylation during a 4 hour stimulus. While not wishing to be bound by any mechanism, and while not proposing any mechanism herein, the results disclosed herein suggest that HDAC inhibitors may favor subsequent rounds of CREB phosphorylation either by promoting association of PKA with acetylated chromatin or by enhancing access of PKA to the chromatin bound CREB.

In vitro reconstitution studies using chromatin assembled templates revealed that periodic nucleosomal arrays can block phosphorylation of the CREB paralog, CREM, by PKA and that this effect can be partially reversed by adding CRE oligonucleotide or by acetylating template-bound nucleosomes with P300 (see Example VI). The inability of P300 to completely restore phosphorylation of CREM by PKA can reflect a less than stoichiometric acetylation of nucleosomes due, for example, to trichostatin A (TSA)-insensitive HDACs in the Drosophila S190 extracts. Nevertheless, the low efficiency of CREM phosphorylation by PKA on unacetylated chromatin templates contrasted sharply with the high stoichiometry of cellular CREB phosphorylation observed during the initial phase of the cAMP response. These results suggest that a substantial pool of CREB in unstimulated cells may be targeted

to acetylated chromatin, or that a pool of CREB may be non-chromosomal, but associates with chromatin subsequent to PKA phosphorylation and CBP recruitment.

- The invention provides a means to alter the expression of a nucleosomal organized nucleic acid molecule by modulating attenuation of expression of the nucleic acid molecule, which contains an expressible nucleotide sequence operably linked to an inducible response element, which is bound in a phosphorylation dependent manner by a response element binding factor. As used herein, the term "alter" or "affect," when used in reference to the expression of a nucleosomal organized nucleic acid molecule, means that the level of expression of the nucleic acid molecule is increased or is decreased.

- Similarly, the term "modulating," when used in reference to attenuation of expression of the nucleic acid molecule, means inhibiting or enhancing attenuation, such that the period of attenuation is increased or is decreased, respectively. By "period of attenuation" is meant the time that it takes for transcription of a gene to go from its maximal level to the time at which it reaches a basal level or other steady state level characteristic of the particular gene. For example, the period of attenuation of expression of a gene that is induced by the cAMP pathway is about 2 to 4 hours. In comparison, a method of the invention that inhibits attenuation of a cAMP inducible gene results in the period of attenuation being longer than about 2 to 4 hours. It follows that an agent that modulates the attenuation of nucleosomal organized nucleic acid molecule can be identified by comparing the period of attenuation of expression of a nucleosomal organized nucleic acid molecule in the presence of the agent with that in the absence of the agent.

- As used herein, the term "nucleosomal organized nucleic acid molecule" means a nucleic acid molecule that is contained in a structure that has or approximates the structure of a nucleosome. Thus, a nucleosomal organized nucleic acid molecule is a nucleoprotein complex that includes a nucleic acid molecule and histone proteins, including histone H4 if desired. For purposes of the present invention, the "nucleic acid molecule" contains an inducible response element and, generally, an expressible nucleotide sequence.

A nucleosomal organized nucleic acid molecule can exist in a cell *in vivo*, for example, a gene normally present in a cell or a gene introduced into a cell by a recombinant or other method and that is integrated into the genome; or can be isolated

5 from a cell or formed *in vitro* using chromatin assembly methods as disclosed herein or otherwise known in the art. For convenience of discussion, the terms "gene" and "nucleosomal organized nucleic acid molecule" are used interchangeably herein, unless indicated otherwise. Whether a nucleic acid molecule is in a nucleosomal organized form can be determined, for example, by performing limited exonuclease

10 digestion and using gel electrophoresis to identify the characteristic ladder of bands, each separated by about 140 to 180 nucleotides, that is formed due to protection from digestion of the nucleotide sequences associated with the histones (see Example VI).

Whether expression from a nucleosomal organized nucleic acid molecule is increased or decreased, and whether attenuation of expression is inhibited or enhanced such that the period of attenuation of expression is increased or decreased, respectively, will depend on the specific manner in which a method of the invention is performed. For example, a method of the invention can be performed by inhibiting deacetylation of the histones involved in forming the structure of the nucleosomal organized nucleic acid molecule or by increasing acetylation of such histones, in which case attenuation is inhibited, the period of attenuation of expression of the nucleic acid molecule is increased, and the level of gene expression is increased.

20 Histone deacetylation can be inhibited by inhibiting the activity of an HDAC using an HDAC inhibitor such as trichostatin A, a butyrate compound, trapoxin, or an anti-

25 HDAC antibody that binds the HDAC and inhibits its deacetylase activity. Alternatively, a method of the invention can be performed by increasing deacetylation of histones using an HDAC inducer, or by decreasing histone acetylation using an HAT inhibitor, thereby enhancing attenuation of modulation of expression of a gene and suppressing gene expression.

30

The present invention further provides a method of ameliorating a pathologic condition by suppressing or potentiating expression of a gene regulated by a phosphorylation dependent response element binding factor in a subject, by

administering to the subject a pharmaceutical composition of the invention. For example, a method of ameliorating a pathologic condition can potentiate gene expression by administering a pharmaceutical composition containing a histone deacetylase (HDAC) inhibitor and an agent that induces expression of the gene 5 through the phosphorylation dependent response element binding factor.

Various pathologic conditions are amenable to treatment, which can be palliative or curative, using a method of the invention. The phosphorylation dependent response element binding factor CREB has been characterized as being an 10 important protein involved in memory and learning in several model systems, including *Aplysia* and rodents. For example, induced expression of a highly active CREB protein in *Drosophila* dramatically improved learning in an olfactory paradigm. In contrast, knockout mice that were deficient in CREB demonstrated impaired learning in a water maze test. Thus, CREB can have a role in memory 15 acquisition and can be useful, for example, for treating subjects that suffer from impaired learning disorders, memory disorders, or other cognitive disorders, including Alzheimer's disease and other dementias such as occur due to aging. Regulation of gene expression by CREB also can be involved in other neuropathologic conditions, including motor disorders such as Parkinson's disease, a neuromuscular pathology 20 such as amyotrophic lateral sclerosis, and a cardiac dysfunction; a renal dysfunction; an endocrine disorder; a dysfunction of gluconeogenesis; an immunosuppressive condition; an osteopathologic condition; a dermal condition, and the like.

Similarly, the Rel family of transcription factors, including NF- κ B, can 25 regulate the expression of viral genes, cytokine genes, growth factor genes and others, which contain a κ response element. As such, a method of the invention can be used, for example, to potentiate expression of a cytokine gene, which is involved in the stimulation of an immune response, and, therefore, can be useful for stimulating a patient's immune response against a particular antigen, for example, a tumor antigen. 30 In performing such a method, the pharmaceutical composition administered to the subject can include an HDAC, an agent that induces expression of the Rel binding factor dependent gene, and the particular antigen against which the immune response

is to be stimulated, thereby stimulating the subject's immune response and ameliorating the pathologic condition.

- As used herein, the term "ameliorating," when used in reference to a
- 5 pathologic condition, means that the clinical symptoms or signs of the pathologic condition are lessened. The skilled artisan will know various methods for determining whether the clinical signs or symptoms of a pathologic condition are ameliorated depending, for example, on the particular pathologic condition being treated. Thus, where a method of the invention is used to treat a subject having a
- 10 cognitive disorder, an appropriate method can include a learning test, a memory test, or the like. Similarly, where the pathologic condition being treated is a cardiac dysfunction, the artisan can perform a treadmill test, or can perform diagnostic tests that measure, for example, serum enzyme levels.
- 15 As used herein, the term "potentiating gene expression" means that transcription of a gene is increased due to inhibiting attenuation of expression of the gene. It should be recognized that the disclosed methods do not necessarily increase the rate of transcription of a gene but, primarily, prolong the time that transcription is maintained at an elevated level by inhibiting attenuation of expression of the gene.
- 20 As such, a method that potentiates expression of a gene regulated by a phosphorylation dependent response element binding factor results in an increased production of the gene product encoded by the gene. Similarly, a method of the invention that "suppresses gene expression" shortens the period of attenuation, thereby decreasing the level of gene expression as compared to expression that would
- 25 occur in the absence of the agent.

A method of ameliorating a pathologic condition as disclosed herein is particularly useful in combination with a method of gene therapy. Gene therapy can be performed (see, for example, U.S. Patent No. 5,399,346) using a nucleic acid

30 molecule containing a selected inducible response element operably linked to a nucleotide sequence encoding a desired gene product. The gene product can be, for example, a cytokine gene product, which can be useful in a gene immunotherapy method; can be a product that corrects a deficiency in a cell, for example, adenosine

deaminase, cystic fibrosis transmembrane receptor, or the like; or can be a toxic agent, for example, ricin, which can kill a cell in which it is expressed.

- The selected inducible response element can be any inducible response element as described herein. The response element can be selected based on the cells in which the gene product is to be, since such cells must express or be capable of expressing the cognate phosphorylation dependent response element binding factor. The gene therapy then is performed under conditions that permit at least relatively stable integration of the nucleic acid construct into the genome of the cell. It should be recognized that the integration need be stable only with respect to the length of time the therapy is to be performed. Thus, where the gene product is a toxin or an immunotherapeutic agent to be used, for example, to treat a cancer, the integration need be stable only for the time until the gene product is expressed using a method of the invention, since the cells then will be killed or otherwise inactivated.

15

A method of the invention then is performed by administering to the subject containing the integrated nucleic acid construct a pharmaceutical composition containing an HDAC inhibitor and the inducing agent specific for the response element that is operably linked to the expressible nucleotide sequence. Such a method allows for selective induction of expression of the gene product and, further, provides potentiated expression by inhibiting attenuation of the expression, thus maximizing expression of the gene product in the cells of interest.

- 25 A method of ameliorating a pathologic condition as disclosed herein is performed by administering to the subject to be treated a pharmaceutical composition, which contains an HDAC inhibitor and an agent that induces expression of a gene through a phosphorylation dependent response element binding factor. One skilled in the art would know that a pharmaceutical composition of the invention can be administered to a subject by various routes including, for example, orally or
- 30 parenterally such as intravenously. The composition can be administered by injection or by intubation. The pharmaceutical composition also can be encompassed within liposomes or other polymer matrices (Gregoriadis, Liposome Technology, Vol. 1 (CRC Press, Boca Raton, FL 1984)). Liposomes, for example, which consist of

phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

An additional advantage of encompassing a pharmaceutical composition of the invention within a liposome or other matrix is that targeting molecules can be incorporated therein. Thus, where it is desired to deliver a pharmaceutical composition of the invention to a particular tissue, a liposome can have incorporated into its bilayer a ligand (or receptor) that binds a cognate receptor (or ligand) expressed exclusively or primarily on the tissue of interest, or an antibody that specifically binds an antigen expressed on the surface of a cell in the target tissue.

For use in the therapeutic methods of the invention, an effective amount of the pharmaceutical composition must be administered to the subject. As used herein, the term "effective amount" means the amount of the HDAC inhibitor and the inducing agent that produces the desired effect, for example, that potentiates gene expression by inhibiting attenuation of expression of the gene. In determining an effective amount to be administered to a subject for a specific purpose, methods well known to those in the art, including phase I and phase II clinical trials, are utilized.

The route of administration of a pharmaceutical composition of the invention will depend, in part, on the chemical structure of the HDAC inhibitor and the inducing agent. Peptides, for example, are not particularly useful when administered orally because they can be degraded in the digestive tract. However, methods for chemically modifying peptides to render them less susceptible to degradation by endogenous proteases or more absorbable through the alimentary tract are well known (see, for example, Blondelle et al., *Trends Anal. Chem.*, 14:83-92 (1995); Ecker and Crook, *Bio/Technology*, 13:351-360 (1995)). In addition, suitable HDAC inhibitors can be identified from libraries of HDAC analogs such as peptides containing D-amino acids; peptidomimetics consisting of organic molecules that mimic the structure of an HDAC; or peptoids such as vinylogous peptoids, using the screening methods disclosed herein.

The invention further provides a method of identifying an agent that modulates attenuation of expression of a nucleic acid molecule, by contacting, under suitable conditions and for an appropriate period of time, a) a test agent, which is suspected of having the ability to modulate attenuation of gene expression, b) a

5 nucleosomal organized nucleic acid molecule containing an expressible nucleotide sequence operably linked to an inducible response element that is regulated by a phosphorylation dependent binding factor, and c) an inducing agent, which induces expression from the response element; and detecting a difference in attenuation of expression of the nucleic acid molecule in the presence of the test agent as compared

10 to attenuation in the absence of the test agent. As used herein, the term "test agent" means a peptide or other organic molecule that is being examined, using a method of the invention, for the ability to modulate attenuation of gene expression.

In general, an identified agent, which modulates attenuation of expression of a

15 nucleosomal organized nucleic acid molecule, does not substantially modulate attenuation of expression of the nucleic acid molecule when it is not in a nucleosomal organized form, and does not substantially affect early burst phase transcription of the nucleic acid molecule. Such characteristics of an identified agent can be confirmed by performing the appropriate control experiments, either in parallel with the

20 screening method, or only on those agents initially identified as having the ability to modulate gene expression.

A screening method of the invention is useful for identifying HDAC inhibitors or HAT inducers, either of which can inhibit the attenuation of expression of a

25 nucleosomal organized nucleic acid molecule and potentiate its expression, or HDAC inducers or HAT inhibitors, either of which can enhance attenuation of expression of a gene. A screening method of the invention can be performed *in vivo* in a cell or *in vitro* using a cell extract and appropriate reagents. For example, a screening method of the invention can be performed using a cell line that is stably transfected with a

30 nucleic acid molecule containing an inducible response element and an expressible nucleotide sequence. An example of such a cell line is disclosed in Example I. Upon contact of such a cell with a test agent and an inducing agent, transcription of the nucleotide sequence can be measured and, specifically, the attenuation phase of

transcription can be measured. Alternatively, the period of phosphorylation of the specific response element binding factor can be measured to determine whether an increased phosphorylation state is maintained beyond the time it normally would decrease (i.e., in the absence of the test agent).

5

A screening assay of the invention also can be performed *in vitro* using a chromatin assembly cell extract (see Example VI). Using such a method, prolonged phosphorylation of a response element binding factor in a chromatin dependent manner can identify a test agent that has the ability to inhibit attenuation of gene expression. The ability of the agent to inhibit attenuation of gene expression can be confirmed using a transcription system as disclosed herein (see Examples I and II).

The screening methods of the invention are useful for high throughput screening of large libraries of diverse molecules. Such libraries, which can be purchased from commercial sources or can be synthesized using well known methods, include, for example, peptide libraries (U.S. Patent No. 5,264,563); peptidomimetic libraries (Blondelle et al., *supra*, 1995); oligosaccharide libraries (York et al., *Carb. Res.*, 285:99-128, (1996); Liang et al., *Science*, 274:1520-1522, (1996); and Ding et al., *Adv. Expt. Med. Biol.*, 376:261-269, (1995)); lipoprotein libraries (de Kruif et al., *FEBS Lett.*, 399:232-236, (1996)); glycoprotein or glycolipid libraries (Karaoglu et al., *J. Cell Biol.*, 130:567-577 (1995)); or chemical libraries containing, for example, drugs or other pharmaceutical agents (Gordon et al., *J. Med. Chem.*, 37:1385-1401 (1994); Ecker and Crooke, *supra*, 1995).

25 The following examples are intended to illustrate but not to limit the invention in any manner, shape, or form, either explicitly or implicitly. While they are typical of those that might be used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

EXAMPLE 1**HISTONE DEACETYLASE INHIBITORS ACT SYNERGISTICALLY WITH cAMP AGONIST TO PROMOTE SOMATOSTATIN mRNA ACCUMULATION**

5 This example demonstrates that histone deacetylase (HDAC) inhibitors promote cAMP induced accumulation of somatostatin mRNA through CREB, but do not enhance CREB activity by stimulating protein kinase A.

10 The role of cellular histone acetyltransferase (HAT) activities in promoting expression of cAMP responsive genes was examined using the stable NIH3T3 cell line D5 ("D5 cells"), which contain rat somatostatin gene sequences from 750 base pairs upstream of the promoter to 3 kilobases downstream of the coding region (Montminy et al., *J. Neurosci.* 6:803-813 (1986)). D5 cells were maintained in Dulbecco's minimal essential medium with 10% calf serum plus 200 µg/ml G418.

15 D5 cells were treated either with vehicle (control), 10µM forskolin, 15 mM sodium butyrate, 100 ng/ml trichostatin A, 100 nM trapoxin, or combinations of these reagents for up to 4 hr. Samples were removed after 0.5, 1, 2 and 4 hr and total RNA was isolated. Northern blot analysis was performed as described previously, using 20 either an antisense RNA probe to rat somatostatin or an α -tubulin cDNA probe (Montminy et al., *supra*, 1986).

25 After incubation for 4 hr, all three HDAC inhibitors, butyrate, trichostatin A, and trapoxin, markedly potentiated somatostatin mRNA accumulation in cells co-stimulated with the cAMP agonist forskolin, but had only modest effects on target gene expression by themselves. Analysis of the time course of mRNA accumulation demonstrated that cooperativity between cAMP and the HDAC inhibitors was greatest at the later times, with optimal synergism at 2 to 4 hr. The HDAC inhibitors also potentiated chromosomal somatostatin gene expression and endogenous c-fos gene 30 expression in other NIH3T3 lines, as well as in clonal isolates of PC12 cells, confirming that the effect observed in D5 cells was not an integration site or cell type selective effect.

cAMP stimulates somatostatin gene expression through the PKA mediated phosphorylation of CREB at Ser133 (Gonzalez and Montminy, Cell 59:675-680 (1989)). To determine whether HDAC inhibitors promote somatostatin gene expression through a CREB dependent mechanism, the effect of expressing dominant negative CREB mutant, A-CREB, was examined (Ahn et al., Mol. Cell Biol. 18:967-977 (1998)).

Approximately 4.4×10^5 cells per 100 mm dish were plated for transient transfection by calcium phosphate coprecipitation technique. For each 100 mm dish, 10 8 µg of each construct (the A-CREB CMV expression vector or a CMV expression vector without insert) plus an RSV-GFP marker was independently co-precipitated with 8 µg pCA-GFP (green fluorescent protein) to select for transfected cells. A total of 10^7 D5 cells per construct was subjected to fluorescence activated cell sorting to obtain an average of 95% pure population of transfected cells. The sorted cells were 15 then treated with various combinations of 10 µM forskolin, 100 ng/ml trichostatin A (BIOMOL Research Laboratories), 15 mM sodium butyrate, or 100 nM trapoxin.

Northern blot analysis revealed that expression of the A-CREB polypeptide potently inhibited somatostatin mRNA accumulation in response to both forskolin and 20 TSA, but had no effect on α-tubulin mRNA levels in the same cells. These results demonstrate that CREB is required for HDAC inhibitors to augment somatostatin gene expression.

To rule out non-specific effects of HDAC inhibitors on the cAMP pathway, 25 cellular PKA activation assays were performed using synthetic Kemptide fragment as a phosphorylation substrate. The cellular PKA activation assay was performed as previously described (Corbin, Meth. Enzymol. 99:227-232 (Academic Press 1983)). D5 cells were stimulated for various times with 10 µM forskolin plus 500 µM isobutyl methyl-xanthine (IBMX), with or without TSA. Cells then were collected and lysed 30 in 100 µl HB (10 mM potassium phosphate, pH 6.8, 1 mM β-mercaptoethanol, 10 µg/ml leupeptin, 10 mM magnesium acetate, 10 µM ATP containing 5×10^5 cpm $\gamma^{32}\text{P}$ -ATP (3000 Ci/mmol), and 30 µg Kemptide substrate. Background was determined from reactions lacking Kemptide substrate and total PKA activity was

estimated in reactions containing 20 μ M dibutyryl cAMP. Reactions were incubated for 5 min at 30 C; aliquots were spotted onto Whatman P-81 paper; and the filters were washed in 75 mM phosphoric acid twice for 1 min each. 32 P incorporation was determined by liquid scintillation counting. Three independent experiments were
5 performed.

Forskolin treatment induced 70% of total cellular PKA activity within 30 min of stimulation, and this effect persisted throughout the 2 hr time course of treatment. Co-stimulation with TSA did not potentiate cellular PKA activity either alone or in
10 combination with forskolin stimulus. Indeed, total levels of PKA catalytic subunit remained constant in TSA+forskolin stimulated cells by western blot assay. These results indicate that HDAC inhibitors do not enhance CREB activity by stimulating PKA.

15

EXAMPLE II

HDAC INHIBITORS STIMULATE CREB ACTIVITY DURING LATE ATTENUATION PHASE OF TRANSCRIPTION IN RESPONSE TO cAMP

This example demonstrates that HDAC inhibitors potentiate gene expression
20 by inhibiting the attenuation phase of cAMP induced transcription.

It has been hypothesized that, following its recruitment to the promoter via Ser133 phosphorylated CREB, the co-activator CBP mediates target gene activation, in part, by acetylating and disrupting promoter-bound nucleosomes (Bannister and
25 Kouzarides, *supra*, 1996; Ogryzko, *supra*, 1996). To determine whether HDAC inhibitors potentiate CREB activity by enhancing nucleosome acetylation over the somatostatin promoter during the burst phase of transcription, chromatin immunoprecipitation assays were performed.

30 D5 cells were grown to near confluence in 15 cm dishes, then treated for 30 min in the absence or presence of forskolin (10 μ M) and/or trichostatin A (100 ng/ml). Cellular histone proteins were crosslinked to chromatinized DNA for 10 min at 37°C by addition of 1% formaldehyde to the medium. Crude nuclei were

isolated by Triton X-100 lysis (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5), resuspended in 0.5 ml SDS/lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.0) and sonicated to reduce the chromatin DNA length to approximately 200-2000 base pairs.

5

The chromatin solution was diluted 10-fold in IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.0, 165 mM NaCl) and incubated with 4 µl anti-acetylated H4 antiserum (Lin et al., *J. Cell Biol.* 108:1577-1588 (1989)). Two µg poly(dI-dC) was added to each IP reaction, and immune complexes were collected with protein A agarose beads. Following extensive washing and elution in 1% SDS/0.1M NaHCO₃, DNA-histone protein crosslinks were reversed by incubation at 65°C for 4 hr. Released DNA was purified by proteinase K digestion, phenol extraction, and ethanol precipitation. Immunoprecipitated DNA was immobilized onto ZETA-PROBE membrane (BioRad) by slot blotting, and somatostatin gene sequences were detected by hybridization with an antisense RNA probe (Braunstein et al., *Genes Devel.* 7:692-604 (1995)). Three independent experiments were performed.

Forskolin treatment had a small but reproducible effect on histone acetylation over the somatostatin promoter after 30 min. In contrast, TSA induced a robust 4-fold increase in nucleosomal acetylation during the same time period, but co-stimulation with forskolin had no greater effect compared to TSA alone.

To evaluate whether the TSA-dependent acetylation of nucleosomes over the somatostatin promoter during the burst phase was sufficient to promote somatostatin gene transcription in D5 cells co-stimulated with cAMP agonist, run-on transcription assays were performed. D5 cells were stimulated for various times from 30 min to 4 hr with 10 µM forskolin and/or 100 ng/ml TSA. Nuclear run-on transcription was performed as previously described using purified cDNA inserts for somatostatin and tubulin (Hagiwara et al., *supra*, 1992). Three independent experiments were performed at the 30 min time point and two independent experiments were performed for the time course.

- Following treatment with forskolin alone, somatostatin transcription rates increased 4-5 fold at 30 min. Remarkably, TSA had no effect on somatostatin gene expression at 30 min, either alone or in combination with forskolin. The lack of cooperativity between TSA and forskolin at this time point did not reflect variable
5 sensitivity of D5 cells to either inducer, since TSA promoted H4 acetylation in greater than 60% of cells by immunofluorescence analysis, and forskolin stimulated Ser133 phosphorylation of CREB in about 90% of cells. Thus, a majority of cells were responsive to both reagents.
- 10 The delayed effects of HDAC inhibitors on somatostatin mRNA accumulation observed in forskolin-treated D5 cells (Example I) prompted an evaluation of somatostatin transcription rates at later times after stimulation. Following treatment with forskolin alone, somatostatin transcription rates returned to pre-stimulus levels by 4 hr. In cells co-stimulated with forskolin plus TSA, however, somatostatin
15 transcription rates remained elevated throughout the attenuation phase. These results indicate that the underlying cooperativity between these inducers reflects a late effect of HDAC inhibitor on CREB activity.

EXAMPLE III

20 HDAC INHIBITORS POTENTIATE TARGET GENE EXPRESSION BY
PROLONGING SER-133 PHOSPHORYLATION OF CREB

This example demonstrates that HDAC inhibitors potentiate target gene expression by prolonging Ser133 phosphorylation of CREB in cells co-stimulated
25 with cAMP agonist.

The importance of Ser133 phosphorylation for transcriptional induction via CREB led to an evaluation of phospho (Ser133) CREB levels in cells stimulated with cAMP agonist and/or HDAC inhibitor. Western blot analysis of phospho(Ser133)
30 CREB and total CREB levels in D5 whole cell extracts was examined following stimulation with forskolin or forskolin plus TSA for various times up to 4 hr.

Upon treatment of D5 cells with forskolin, both CREB and its mammalian paralog ATF-1 were phosphorylated to maximal levels after 30 min. Reflecting the previously noted action of the Ser/Thr phosphatase PP-1 on both activators (Hagiwara et al., *supra*, 1992), levels of phospho-ATF-1 and phospho-CREB were diminished in 5 parallel with target gene expression rates during the subsequent attenuation phase. TSA alone had no effect on CREB Ser133 phosphorylation during the 4 hr assay period. When added in combination with forskolin, however, TSA strongly enhanced both CREB and ATF-1 phosphorylation in cells at 2 to 4 hr. Comparable results were observed by immunofluorescence analysis of D5 cells with phospho-CREB specific 10 antiserum.

Nuclear localization of phospho (Ser133) CREB was examined in cells co-stimulated with forskolin plus TSA. D5 cells were treated with activators for varying lengths of time and were lysed with SDS-urea lysis buffer. Whole cell lysates 15 (20 µg) were resolved by 10% SDS-PAGE, and duplicate blots were probed with either an anti-CREB antibody (244) or a phospho (Ser133) specific antiserum (5322; Hagiwara et al., *supra*, 1993). Immune complexes were detected by chemiluminescence. At least five independent experiments were performed for phospho CREB analysis.

20 Immunofluorescence assays were performed as previously described (Alberts et al., *J. Biol. Chem.* 269:7623-7630 (1994)). Immunofluorescence microscopy was performed using phospho (Ser133) CREB specific antiserum 5322 on D5 cells following 2 hr of stimulation with forskolin or forskolin plus TSA.

25 Nuclear phospho(Ser133) CREB staining was virtually undetectable after 2 hr stimulation with forskolin alone, but remained elevated in cells exposed to forskolin plus TSA. Western blot analysis also revealed that trapoxin (100 nM) and sodium butyrate (15 mM) also promoted CREB phosphorylation at late times in cells 30 co stimulated with forskolin, as well as Ser64 phosphorylation of ATF-1 at 2 to 4 hr following co-stimulation with forskolin.

EXAMPLE IV**cAMP AGONIST IS REQUIRED CONTINUOUSLY FOR
POTENTIATION BY HDAC INHIBITORS**

5 In view of the importance of PP-1 in attenuating CREB activity at late times after cAMP induction, the effect of HDAC inhibitors on PP-1 phosphatase activity was examined. Using pulse-chase studies, D5 cells were stimulated with forskolin or forskolin plus TSA for 30 min, then transferred to control medium or medium supplemented with TSA for various times up to 2 hr. Phospho (Ser133) CREB and 10 total CREB levels were examined in D5 whole cell extracts by western blot analysis.

Following removal from the forskolin stimulus, CREB was de-phosphorylated with an estimated t½ of 30 min. TSA had no effect on cellular levels of phospho (Ser133) CREB during the chase period, indicating that PKA activity is continuously 15 required for potentiation via HDAC inhibitors.

An *in vitro* CREB phosphatase assay was performed using ³²P-labeled phospho (Ser133) CREB plus nuclear extracts from control D5 cells or D5 cells treated with forskolin, TSA, or forskolin plus TSA for 2 hr. Extracts (50µg) were 20 incubated with ³²P-labeled CREB at 37°C for times indicated, and samples were visualized by SDS-PAGE. CREB phosphatase activity in control and TSA-treated cells appeared comparable by *in vitro* phosphatase assay with Ser133 phosphorylated CREB.

25 These results indicate that HDAC antagonists do not prolong Ser133 phosphorylation of CREB by inhibiting cellular PP-1 activity, and that cAMP agonist is continuously required for potentiation via HDAC inhibitors.

EXAMPLE V

PERSISTENT ACTIVATION OF NON-CHROMATIN-BOUND CREB
ACTS VIA A CHROMATIN DEPENDENT MECHANISM FOR
TRANSCRIPTIONAL ATTENUATION

5

Nucleosome acetylation enhances accessibility of nuclear factors and other regulatory factors to the mononucleosome (Workman and Kingston, *Ann Rev. Biochem.* 67:545-579 (1998)). To evaluate whether acetylation of nucleosomes in response to HDAC inhibitors similarly increases accessibility of the signaling

10 machinery to chromatin bound CREB polypeptides, CREB activity was compared on chromosomal and plasmid templates, which assemble incompletely into chromatin structures and promote transcription via chromatin independent mechanisms (Jeong and Stein, *Nucl. Acids Res.* 22:370-375 (1994)).

15 Transient transfection assays were performed using 2×10^5 D5 cells plated into 6-well dishes. Each transfection contained 1 μ g CRE-CAT reporter plasmid and 50 ng RSV- β GAL. At least 50% of cells were transfected based on fluorescence from a co-transfected RSV-GFP expression vector. Chloramphenicol acetyl transferase and β -galactosidase assays were performed. GAL4-CREB 1-283 plasmid (8 μ g/100 mm dish) was transfected into D5 cells by calcium phosphate transfection as described (Ahn et al., *supra*, 1998). Following transient transfection, the somatostatin reporter plasmid was induced 14-fold by forskolin after 4 hr. In contrast, HDAC inhibitors had no effect on the extra-chromosomal somatostatin template, either alone or in combination with cAMP agonist.

25

The unresponsiveness of the extra-chromosomal somatostatin template to HDAC inhibitor led to an examination of the phosphorylation status of non-chromatin bound CREB polypeptides as compared to the chromatin-bound population. Following transfection, the GAL4-CREB construct (CREB amino acids 1-283), which 30 lacks the CREB DNA binding domain (amino acids 284-341) and, therefore, is unable to bind to cellular CRE sites, was continuously phosphorylated in response to forskolin treatment, with no discernable attenuation after 4 hr. Co-stimulation of the transfected D5 cells with TSA had no effect on Ser133 phosphorylation of

GAL4-CREB, but potentiated Ser133 phosphorylation of endogenous CREB and Ser64 phosphorylation of the paralog ATF-1 proteins in the same cells. These results indicate that HDAC inhibitors potentiate cAMP induced cellular gene expression by enhancing phosphorylation of CREB and ATF-1 in a chromatin dependent manner.

5

Based on the ability of A-CREB to heterodimerize with and block binding of CREB to cellular CRE sites (Ahn et al., *supra*, 1998), the ability of over-expressed A-CREB dominant negative polypeptide to alter the status of Ser133 phosphorylation, for example, by liberating chromatin bound CREB and, thereby, increasing PKA accessibility, was examined. Following forskolin treatment of control D5 cells transfected with a control dominant negative A-fos plasmid (Olive et al., *J. Biol. Chem.* 272:18586-18594 (1997)), phospho (Ser133) CREB levels were maximal after 10 30 min, then returned to baseline within 3 hr.

15

In cells expressing the A-CREB polypeptide, CREB (Ser 133) phosphorylation was markedly enhanced, both under basal conditions and after prolonged treatment with forskolin. CREB remained heavily phosphorylated throughout the attenuation phase in A-CREB expressing cells (1-3 hr), underscoring the potential importance of chromatin localization for stimulus-appropriate regulation 20 of CREB by PKA. These results demonstrate that over-expression of dominant negative A-CREB polypeptide enhanced phosphorylation of CREB in D5 cells stimulated with forskolin.

EXAMPLE VI

25

IN VITRO CHROMATIN ASSEMBLY ASSAY

The ability of A-CREB to enhance CREB phosphorylation status suggested that promoter bound nucleosomes may repress target gene transcription during the attenuation phase by blocking accessibility of PKA to its substrate. Drosophila 30 chromatin assembly extract (S-190; Kraus and Kadonaga, *supra*, 1998) were used to assemble periodic nucleosomal arrays on a cAMP responsive template, and PKA phosphorylation of chromatin-bound CREM, a CREB paralog that is readily purified to homogeneity in recombinant form, was examined. Under these conditions, CREM

and CREB bind CRE sites on chromatin assembled templates in a phosphorylation independent manner (Mayall et al., *supra*, 1997).

Chromatin assembly and analysis was performed using the plasmid, 3X CRE-
5 MLP (Nakajima et al., *supra*, 1997), which contains three CRE sites inserted upstream of the Adenovirus major late promoter. Drosophila S190 extract was preincubated with purified Drosophila core histones for 30 min at room temperature (Kraus and Kadonaga, *supra*, 1998), then 500 ng of 3X CRE-MLP plasmid (1.3 nM final) was added to the assembly mixture along with an ATP generating system, and
10 the reaction was incubated for 3.5 hr. at 27 °C. One-dimensional chloroquine supercoiling gel electrophoresis of aliquots of the chromatin assembly reactions confirmed nucleosome deposition on the 3X CRE-MLP template, micrococcal nuclease digestion of the chromatin assembly reactions demonstrated the regularity of nucleosome spacing on the template.

15

Following assembly, CREM phosphorylation assays were performed. For phosphorylation experiments, 3.9 nM CREM was incubated with the chromatin template for 30 min at 27°C, to allow CREM to bind to its target sites, then approximately 12 nM PKA catalytic subunit was added to each reaction. In some
20 experiments, 100-fold molar excess CRE was added along with CREM to inhibit template binding. In some reactions, P300 (200 nM) was added along with TSA (100 ng/m1) and acetyl CoA to promote acetylation of the chromatin template. In time-course experiments, aliquots of the reactions were stopped by mixing with 2X SDS loading buffer. Phospho-CREM levels were assessed by immunoblotting
25 with phospho-specific antiserum. Comparable recovery of total CREM from each reaction was verified by western blot analysis using a the non-discriminating antiserum 244.

PKA phosphorylation of CREM was severely attenuated with chromatin
30 templates, but not with non-chromatin templates derived from identical assembly reactions lacking histones; there was virtually no increase in phosphorylation of CREM after 30 min incubation with the chromatin template. S190 extract alone induced low level phosphorylation of CREM at Ser71 during chromatin assembly;

and addition of PKA catalyzed Ser71 phosphorylation 2.5 fold over baseline on non-chromatin templates. Addition of CRE oligonucleotide in 100-fold molar excess (400 nM) relative to CRE sites on the chromatin template promoted phosphorylation of CREM by PKA 2-fold over baseline, confirming that binding of CREM to
5 chromatinized template is necessary to block accessibility to PKA.

The importance of CBP/P300 HAT activity for activation of cAMP responsive genes prompted an examination of whether P300 could reverse the inhibitory effects of chromatin on PKA mediated phosphorylation of CREM. When added to chromatin
10 assembly reactions, P300 (200 nM) partially rescued PKA mediated Ser71 phosphorylation of CREM, suggesting that nucleosome disruption is sufficient to enhance accessibility of PKA to its target substrates. These results indicate that the phosphorylation status of a signal dependent activator is in part determined by the configuration of the surrounding chromatin. The results disclosed herein demonstrate
15 that HDAC inhibitors inhibit the attenuation of cAMP inducible gene expression, in a nucleosomal dependent manner, by prolonging the period of time that CREB is phosphorylated in response to the inducing agent.

It will be apparent to those skilled in the art that various modifications and
20 variations can be made to the compositions and processes of this invention.
Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A composition, comprising a first agent that affects histone acetylation and a second agent that induces gene expression through a phosphorylation dependent response element binding factor.
2. The composition of claim 1, wherein the first agent is a histone deacetylase (HDAC) inhibitor.
3. The composition of claim 2, wherein the HDAC inhibitor is selected from the group consisting of trichostatin A, a butyrate compound, and trapoxin.
4. The composition of claim 2, wherein the HDAC inhibitor is an anti-HDAC antibody, or an HDAC binding fragment of said antibody.
5. The composition of claim 1, wherein the response element binding factor is a transcription factor.
6. The composition of claim 1, wherein the response element binding factor is a cyclic adenosine 3', 5'-monophosphate (cAMP) response element binding factor (CREB).
7. The composition of claim 1, wherein said second agent is a second messenger or derivative thereof.
8. The composition of claim 1, wherein the second agent is selected from the group consisting of calcium ion, a neurotrophic factor, a growth factor, a hormone, a physical stimulus, a chemical stimulus, and an environmental stimulus.
9. The composition of claim 1, wherein the second agent induces activity of a protein kinase, which phosphorylates the phosphorylation dependent response element binding factor.

10. The composition of claim 1, wherein the second agent induces activity of a protein phosphatase, which dephosphorylates the phosphorylation dependent response element binding factor.
11. The composition of claim 1, wherein the first agent a HDAC inducer.
12. The composition of claim 1, wherein the first agent is selected from the group consisting of a histone acetylase (HAT) inhibitor and an HAT inducer.
13. The composition of claim 1, which is a pharmaceutical composition.
14. A method of modulating the phosphorylation state of a phosphorylation dependent response element binding factor, comprising contacting a cell containing the binding factor with an agent that affects histone acetylation, thereby modulating the phosphorylation state of the phosphorylation dependent response element binding factor.
5
15. The method of claim 14, wherein the agent that affects histone acetylation is a histone deacetylase (HDAC) inhibitor.
16. The method of claim 15, wherein the HDAC inhibitor is selected from the group consisting of trichostatin A, a butyrate compound, and trapoxin.
17. The method of claim 14, further comprising contacting the cell with an agent that induces gene expression through the phosphorylation dependent response element binding factor.
18. The method of claim 17, wherein the agent induces expression of a protein kinase, which phosphorylates the response element binding factor.
19. The method of claim 17, wherein the agent induces expression of a protein phosphatase, which dephosphorylates the response element binding factor.

20. The method of claim 17, wherein the agent is a second messenger.
21. The method of claim 14, wherein the response element binding factor is a cyclic adenosine 3', 5'-monophosphate (cAMP) response element binding factor (CREB).
22. The method of claim 21, further comprising contacting the cell with an agent that induces gene expression through CREB.
23. The method of claim 22, wherein the agent is a cell permeable derivative of cAMP.
24. The method of claim 22, wherein the agent increases the level of cAMP in the cell.
25. The method of claim 24, wherein the agent is forskolin.
26. The method of claim 22, wherein the agent is selected from the group consisting of calcium ion, a neurotrophic factor, a growth factor, a hormone, a physical stimulus, a chemical stimulus, and an environmental stimulus.
27. The method of claim 14, wherein the phosphorylation dependent response element binding factor comprises a polypeptide or polypeptide subunit selected from the group consisting of serum response element binding factor, activator protein-1, c-jun, v-jun, c-fos, v-fos, Elk, signal transducer and activator of transcription-1, nuclear factor of activated T lymphocytes, a ternary complex factor, E2F, DP1; CCAAT enhancing binding protein, an activating transcription factor), nuclear factor- κ B and a Rel family member.
5
28. A method of ameliorating a pathologic condition by potentiating expression of a gene regulated by a phosphorylation dependent response element binding factor in a subject, comprising administering to the subject the pharmaceutical composition of claim 13.

29. The method of claim 28, wherein the second agent is an HDAC inhibitor.
30. The method of claim 29, wherein the HDAC inhibitor is selected from the group consisting of trichostatin A, a butyrate compound, and trapoxin.
31. The method of claim 29, wherein the HDAC inhibitor is an antibody that specifically binds an HDAC, or an HDAC binding fragment of said antibody.
32. The method of claim 28, wherein the second agent is a second messenger.
33. The method of claim 28, wherein the response element binding factor is a cyclic adenosine 3',5'- monophosphate (cAMP) response element binding factor (CREB).
34. The method of claim 33, wherein the second agent is selected from the group consisting of a cell permeable analog of cAMP and forskolin.
35. The method of claim 28, wherein the pathologic condition is selected from the group consisting of a neuropathology and a neuromuscular pathology.
36. The method of claim 35, wherein the pathology is a neuropathology, which results in a cognitive disorder.
37. The method of claim 36, wherein the cognitive disorder is Alzheimer's disease.
38. The method of claim 35, wherein the neuropathology is selected from the group consisting of Parkinson's disease and amyotrophic lateral sclerosis.
39. The method of claim 28, wherein the pathologic condition is selected from the group consisting of a cardiac dysfunction, a renal dysfunction; an endocrine disorder; a dysfunction of gluconeogenesis; an immunosuppressive condition; an osteopathologic condition; and a dermal condition.

40. A method of altering expression of a nucleosomal organized nucleic acid molecule, comprising modulating attenuation of expression of the nucleic acid molecule,

5 wherein said nucleic acid molecule comprises an inducible response element and an expressible nucleotide sequence, and

wherein said inducible response element is bound in a phosphorylation dependent manner by a response element binding factor.

41. The method of claim 40, comprising affecting acetylation of histones comprising the nucleosomal organized nucleic acid molecule, thereby inhibiting attenuation of expression of the nucleic acid molecule.

42. The method of claim 41, wherein affecting acetylation of histones comprises inhibiting histone deacetylation.

43. The method of claim 42, wherein inhibiting deacetylation of histones is mediated by an HDAC inhibitor.

44. The method of claim 41, comprising inhibiting acetylation of histones comprising the nucleosomal organized nucleic acid molecule, enhancing attenuation of expression of the nucleic acid molecule.

45. A method of identifying an agent that modulates attenuation of expression of a nucleic acid molecule, comprising the steps of:

a) contacting a test agent;

5 a nucleosomal organized nucleic acid molecule, wherein said nucleic acid molecule comprises an inducible response element operably linked to an expressible nucleotide sequence, and wherein expression from said inducible regulatory element is regulated by a phosphorylation dependent response element binding factor; and

10 an inducing agent, which induces expression from the response element; and

- b) detecting a difference in attenuation of expression of the nucleic acid molecule in the presence of the test agent as compared to attenuation in the absence of the test agent, thereby identifying an agent that modulates attenuation of expression of the nucleosomal organized nucleic acid molecule;
- 15 wherein the agent that modulates attenuation of expression does not substantially modulate attenuation of expression of a non-nucleosomal organized nucleic acid molecule and does not substantially affect early burst phase transcription of the nucleic acid molecule.
46. The method of claim 44, wherein the agent that modulates attenuation of expression inhibits the attenuation of expression of the nucleic acid molecule.
47. The method of claim 44, wherein the agent is a histone deacetylase inhibitor.
48. The method of claim 44, wherein the agent is a histone acetylase inducer.
49. The method of claim 44, wherein the agent that modulates attenuation of expression enhances the attenuation of expression of the nucleic acid molecule.
50. The method of claim 48, wherein the agent is a histone deacetylase inducer.
51. The method of claim 44, wherein the agent is a histone acetylase inhibitor.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/24211

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/00; C12N 5/02; C12Q 1/68
US CL : 435/6, 375; 514/1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 375; 514/1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JIN et al. Transcriptional Regulation of the MDR1 Gene by Histone Acetyltransferase and Deacetylase Is Mediated by NF-Y. Molecular and Cellular Biology. July 1998. Vol. 18, No. 7, pages 4377-4384, see entire document.	14-16, 27, 40-44 -----
Y	SALMINEN et al. Neuronal apoptosis induced by histone deacetylase inhibitors. Molecular Brain Research. 1998. Vol. 61, pages 203-206, see entire document.	1-13, 17-26, 28-39, 45-51
X	HAGIWARA et al. Transcriptional Attenuation Following cAMP Induction Requires PP-1-Mediated Dephosphorylation of CREB. Cell. 10 July 1992. Vol. 70, pages 105-113, see entire document.	14-16, 27, 40-44 -----
Y		1-13, 17-26, 28-39, 45-51

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier document published on or after the international filing date
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"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed
"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/24211

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	OGRYZKO et al. The Transcriptional Coactivators p300 and CBP Are Histone Acetyltransferases. Cell. 29 November 1996. Vol. 87, pages 953-959, see entire document.	1-13, 17-26, 28-39, 45-51
Y	NICHOLS et al. Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription. The EMBO Journal. 1992. Vol. 11, No. 9, pages 3337-3346, see entire document.	1-13, 17-26, 28-39, 45-51
Y	US 5,834,249 A (FURUKAWA et al) 10 November 1998, see entire document.	1-13, 17-26, 28-39, 45-51
Y	US 5,939,455 A (REPHAEILI) 17 August 1999, see entire document.	1-13, 17-26, 28-39, 45-51
Y	US 5,710,176 A (REPHAEILI et al) 20 January 1998, see entire document.	1-13, 17-26, 28-39, 45-51
Y, P	US 5,972,608 A (PETERSON et al) 26 October 1999, see entire document.	45-51

INTERNATIONAL SEARCH REPORT

International application No.
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B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

West, Dialog OneSearch (biotech databases)

search terms: hdac, histone deacetylase, phosphor?, creb, camp, tsa, trichostatin A, butyrate, butyric, trapoxin, regulat?, affect?, control?, alter?, inhibit?, stimulat?, phosphatase, hat, induc?, forskolin, pharmaceutical?, treat?, administer?, neuro?, parkinson?, amyotrophic lateral sclerosis, modulat?, assay?